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Chemoenzymatic Synthesis of Glutamic Acid Analogues: Substrate Specificity and Synthetic Applications of Branched Chain Aminotransferase from *Escherichia coli*

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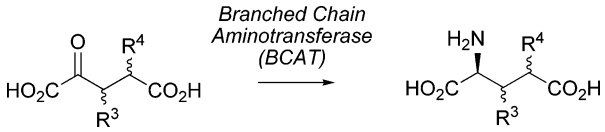
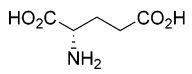


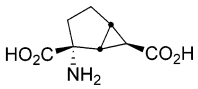
A new route to α-keto acids is described, based on the ozonolysis of enol acetates obtained from α-substituted β-keto esters. *Escherichia coli* branched chain aminotransferase (BCAT) activity toward a variety of substituted 2-oxoglutaric acids was demonstrated analytically. BCAT was shown to have a broad substrate spectrum, complementary to that of aspartate aminotransferase, and to offer access to a variety of glutamic acid analogues. The usefulness of BCAT was demonstrated through the synthesis of several 3- and 4-substituted derivatives.

Introduction

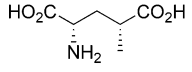
Glutamic acid is the major excitatory neurotransmitter within the central nervous system of vertebrates, where numerous Glu receptors and transporters have been identified.[1−5] Glu analogues behaving as selective ligands are useful tools for elucidating the specific roles of these receptors. Glu analogues may also have some therapeutic effects on several mental disorders where the glutamatergic system is implicated:[3,6−10] for example, the constrained bicyclic LY354740 is an agonist of group II metabotropic Glu receptors and has been recognized as a lead structure for the development of anxiolytics.



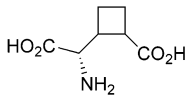
Glu



LY354740



(2S,4R)-4-methylGlu



L-CBG-I to IV

Simple changes in Glu structure can afford some binding selectivity as exemplified by (2*S*,4*R*)-4-methyl Glu, which is a potent selective agonist of kainate receptors.[10] Glu analogues are also of major interest in biologically active peptide mimics.[11,12] Finally, as Glu and pyro-Glu are commonly used as starting material for enantioselective total syntheses, the addition of substituents and chiral centers on the Glu skeleton offers valuable new synthons as exemplified by the recent synthesis of new thalidomide derivatives.[13]

In that context, we have been developing a chemoenzymatic approach for the synthesis of Glu analogues for several years. It is based on the use of aspartate aminotransferase (AAT) for the stereoselective conversion into Glu analogues of substituted α-ketoglutaric acid (KG) readily prepared by chemical synthesis.[14−18] AAT has been shown to be a broad substrate spectrum

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(2) Hertz, L. *Neurochem. Int.* **2006**, *48*, 416−425.
(3) Kew, J. N. C.; Kemp, J. A. *Psychopharmacology* **2005**, *179*, 4−29.
(4) Bridges, R. J.; Esslinger, C. S. *Pharmacol. Ther.* **2005**, *107*, 271−285.
(5) Shigeri, Y.; Seal, R. P.; Shimamoto, K. *Brain Res. Rev.* **2004**, *45*, 250−265.
(6) Foster, A. C.; Kemp, J. A. *Curr. Opin. Pharmacol.* **2006**, *6*, 7−17.
(7) Gardoni, F.; Di Luca, M. *Eur. J. Pharm.* **2006**, *545*, 2−10.
(8) Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. O.; Madsen, U.; Krogsgaard-Larsen, P. *J. Med. Chem.* **2000**, *43*, 2609−2645.
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(11) Mutucumarana, V. P.; Acher, F.; Straight, D. L.; Jin, D.-Y.; Stafford, D. W. *J. Biol. Chem.* **2003**, *278*, 46488−46493.
(12) Milne, C.; Powell, A.; Jim, J.; AlNakeeb, M.; Smith, C. P.; Micklefield, J. *J. Am. Chem. Soc.* **2006**, *128*, 11250−11259.
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(14) Passerat, N.; Bolte, J. *Tetrahedron Lett.* **1987**, *28*, 1277−1280.

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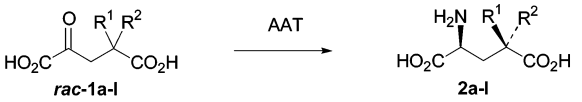
SCHEME 1. AAT-Catalyzed Synthesis of Glutamic Acid Analogues



	a	b	c	d	e	f	g	h	i	j	k	l
R^1	OH	H	Me	Et	Pr	Bu	Pn	iPr	iBu	iPn	Bn	Me	Me
R^2	H	OH	H	H	H	H	H	H	H	H	H	Me	OH

enzyme allowing the preparation of a variety of 4-substituted Glu analogues. As depicted in Scheme 1, AAT from pig heart or *E. coli* was used for the preparation of both isomers of L-4-hydroxy Glu,[14] of a variety of L-2,4-*syn*-4-alkyl Glu[15] as well as 4,4-disubstituted derivatives.[16] Unfortunately, AAT is inactive toward 3-substituted derivatives with the exception of the 3-methyl analogue which is a moderately good substrate. Recently, the scope of AAT was extended to the constrained cyclobutane Glu analogues (L-CBG): three stereomers L-CBG-II to IV were prepared with AAT while the fourth isomer L-CBG-I was obtained using another enzyme: branched chain aminotransferase (BCAT) from *Escherichia coli*.[17,18] BCAT accepts Glu as the amino donor in the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine. BCAT has also been shown to be active toward phenylalanine and methionine. It is thus a broad spectrum enzyme and has been previously used in the preparation of several unnatural aminoacids[19] including L-*tert*-leucine, present in several biologically active pseudopeptides.

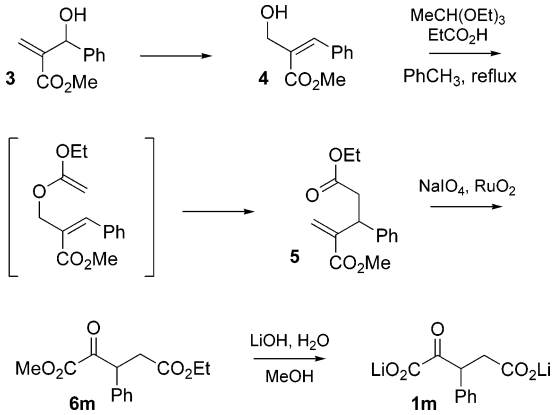
We present here the chemical synthesis of several KG analogues, an analytical study of BCAT substrate specificity, and the preparation of Glu analogues using BCAT and AAT.

Results and Discussion

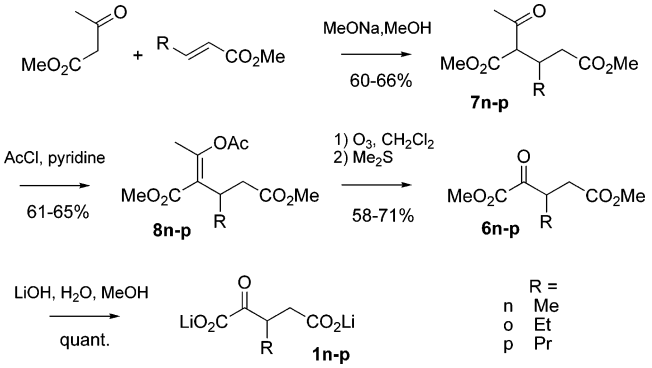
Synthesis of 3-Substituted 2-Oxoglutaric Acids. 3-Phenyl KG **1m** was first prepared according to Scheme 2 using a previously described method based on the Claisen−Johnson rearrangement.[15]

Allylic alcohol **4** was readily prepared following a described procedure including a thermodynamically favored rearrangement of the Baylis−Hillman adduct **3** formed from methyl acrylate and benzaldehyde.[20] The conversion of **4** into **1m** was done with a good overall yield of 56%. This methodology was previously shown to be very efficient for preparing 4-substituted KG from various orthoesters.[15] However, with the exception of compound **4**, the various allyl alcohols needed for the synthesis of 3-substituted derivatives are not easily prepared, and another strategy was therefore developed as shown in Scheme 3. The Michael addition of acetoacetate onto substituted acrylates was performed in basic conditions to afford acetyl glutarates **7n−p**. These β-keto esters were then converted to

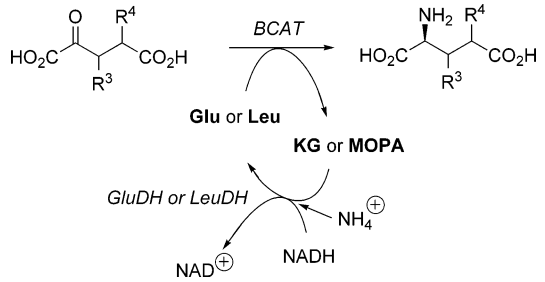
SCHEME 2. Synthesis of 3-Phenyl-KG 1m



SCHEME 3. Synthesis of KG Analogues by Ozonolysis of Enol Acetates



SCHEME 4. BCAT Activity Assay



their enol acetates **8n−p** using AcCl and pyridine. Ozonolysis of the compounds **8** then gave the α-ketoesters **6n−p** that were finally converted to the lithium salts isolated in overall yield of 21−30%.

The formation of the enol acetate intermediates **8** proved to be necessary: attempts to achieve the direct oxidative cleavage of the enolates formed from compounds **7** in basic medium resulted in low yields (<10%) of α-keto ester, while α-hydroxylation of the β-ketoesters **7** was always the main reaction observed (data not shown).

The new synthetic method described here for compounds **1n−p** appears very general and should provide access to a variety of new KG analogues as well as to other α-keto acids in the near future.

Substrate Specificity of BCAT. Kinetic constants for the transamination reaction of KG were determined according to Scheme 4.

Leucine (Leu) was used as the amino donor substrate in quasi-saturating concentration (40 mM). 4-Methyl-2-oxopentanoic acid

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(16) Helaine, V.; Rossi, J.; Gefflaut, T.; Alaux, S.; Bolte, J. *Adv. Synth. Catal.* **2001**, *343*, 692−697.
(17) Gu, X.; Xian, M.; Roy-Faure, S.; Bolte, J.; Aitken, D. J.; Gefflaut, T. *Tetrahedron Lett.* **2006**, *47*, 193−196.
(18) Faure, S.; Jensen, A. A.; Maurat, V.; Gu, X.; Sagot, E.; Aitken, D. J.; Bolte, J.; Gefflaut, T.; Bunch, L. *J. Med. Chem.* **2006**, *49*, 6532−6538.
(19) Hwang, B. Y.; Cho, B. K.; Yun, H.; Koteshwar, K.; Kim, B. G. *J. Mol. Catal. B: Enzym.* **2005**, *37*, 47−55.
(20) Mason, P. H.; Emslie, N. D. *Tetrahedron* **1994**, *50*, 12001.

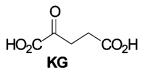
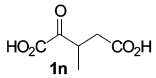
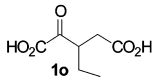
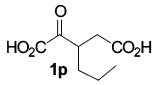
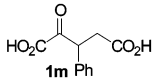
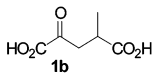
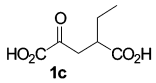
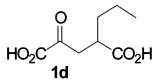
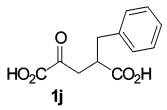
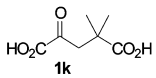
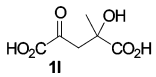
(MOPA) was reduced back to Leu in a non-rate-limiting step in the presence of NADH, NH_4^+, and leucine dehydrogenase (LeuDH). The optical density decrease consecutive to NADH oxidation was measured at 340 nm to monitor the transamination reaction. Rate measurements at various substrate concentrations made it possible to estimate an apparent Michaelis constant (K_m) and maximal velocity ($V_m = k_{cat}$[BCAT]) in the defined experimental conditions on the basis of Michaelis−Menten model for enzyme-catalyzed reactions. In the case of the non-natural KG analogues, the same method was used, but Glu and glutamic dehydrogenase (GluDH) were used instead of Leu and LeuDH. Indeed, GluDH proved to be a much cheaper enzyme and showed no activity toward any KG analogues. For every substrate studied, a titration was performed with the same enzymatic system to provide evidence of a possible enzyme enantiopreference: NADH consumption corresponding to half of the theoretical limiting concentration of KG analogue would indicate that only one enantiomer within the racemate is a good substrate for BCAT. Obviously, this method was appropriate only to demonstrate a high enantioselectivity coefficient ($E >$ 50). Results are reported in Table 1.

BCAT displays a markedly high activity with 3-methyl KG **1n**. Despite a drop in affinity and k_{cat} when the substituent size increases, even the bulky 3-phenyl derivative **1m** remains a good substrate with relative k_{cat} of 75%. Enantioselectivity was shown only for 3-propyl and 3-phenyl KG **1p** and **1m**, whereas complete conversion of racemic 3-methyl and 3-ethyl KG **1n** and **1o** was observed. 4-Alkyl derivatives are also very good substrates of BCAT with, again, a negative effect of the substituent bulkiness. No enantiopreference was observed for any 4-alkyl substrates. Finally, 4,4-disubstituted KG analogues **1k** and **1l** are readily converted to Glu analogues, again without enantioselectivity toward **1l**.

These results contrast to those previously obtained with AAT, which showed good activity only with 4-alkyl KG, stereoselectively converted into L-2,4-*syn*-4-alkyl Glu.[15] *E. coli* BCAT thus appears as a broader spectrum enzyme allowing access to various L-Glu analogues including 3-substituted Glu, as well as L-2,4-*anti*-4-alkyl Glu or (2*S*,4*R*)-4-hydroxy-4-methyl Glu that could not be prepared previously with AAT. In the case where BCAT shows no enantiopreference, the diastereomeric products should be separable, or alternatively, an enantiopure keto acid can be used as the substrate of the transamination reaction, as shown in a following paragraph.

Preparation of Glu Analogues. To confirm the analytical data relative to BCAT activity and enantioselectivity, preparative scale transamination reactions were done on a millimole scale. As transamination reactions are characterized by equilibrium constants near unity, large scale reactions require an equilibrium shift strategy. AAT offers an opportunity through the use of cysteine sulfinic acid (CSA), a close analogue of Asp, as the amino donor substrate: CSA is converted into pyruvyl sulfinic acid, which spontaneously decomposes into pyruvic acid, which is not a substrate for AAT.[21] As CSA is not a substrate of BCAT, another equilibrium shift strategy had to be found. Three different procedures were used with various KG analogues **1**. In all cases, Glu or Leu was used as a catalytic amino donor substrate (0.1 molar equiv), and an irreversible regeneration system allowed the equilibrium shift. Glu was preferably regenerated in a coupled transaminase system using AAT and CSA as the primary stoichiometric amino donor (Scheme 5). Alternatively, GluDH, NH_4^+, and NADH were also used for Glu regeneration. NADH itself was used in a catalytic amount and regenerated using the irreversible oxidation of HCO_2^- ions catalyzed by formate dehydrogenase (FDH) as shown in Scheme 6.[22] Finally, when used as the catalytic donor, Leu was regenerated using the same procedure, using LeuDH in place of GluDH. The choice of the transamination procedure was dictated by purification constraints. Indeed, 3-methyl or 3-ethyl

TABLE 1. Kinetic Parameters of BCAT-Catalyzed Transaminations with KG Analogues[a]

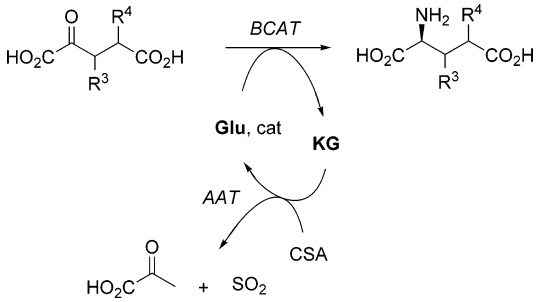
Substrate	K_m *(mM)*	k_{cat} *rel.(%)*	k_{cat}/K_m *rel.(%)*	E [c]
 KG	0,23 ± 0,04	100 [b] ± 3	100 ± 18	
 1n	0,22 ± 0,06	349 ± 29	242 ± 123	-
 1o	0,42 ± 0,05	205 ± 10	76 ± 33	-
 1p	1,9 ± 0,4	93 ± 12	8 ± 4	+
 1m Ph	1,4 ± 0,3	75 ± 9	8 ± 4	+
 1b	0,18 ± 0,03	246 ± 15	211 ± 100	-
 1c	0,26 ± 0,12	220 ± 15	130 ± 83	-
 1d	0,68 ± 0,06	231 ± 12	52 ± 23	-
 1j	0,81 ± 0,07	64 ± 3	12 ± 5	-
 1k	0,20 ± 0,03	166 ± 10	127 ± 57	
 1l	0,58 ± 0,09	262 ± 15	70 ± 32	-

[a] Values and standard errors were calculated from the Hanes−Woolf plot according to the least-squares method and Gauss's error propagation law. [b] The absolute k_{cat} value measured in our experimental conditions was 345 ± 10 min^{-1}. [c] Enantioselectivity toward racemic KG substrate.

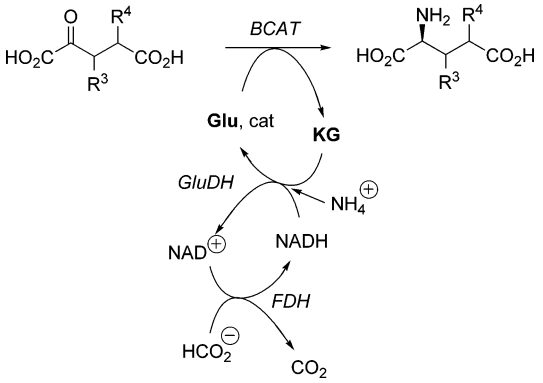
(21) Jenkins, W. T.; D'Ari, L. *Biochem. Biophys. Res. Commun.* **1966**, *22*, 376−82.

(22) Tishkov, V. I.; Popov, V. O. *Biomol. Eng.* **2006**, *23*, 89−110.

SCHEME 5. Equilibrium Shift of BCAT Transamination Using AAT and CSA



SCHEME 6. Equilibrium Shift of BCAT Transamination with GluDH and FDH



Glu **2n** and **2o** proved difficult to separate from Glu. Leu was thus preferred as the amino donor for the synthesis of both derivatives. In contrast, **2p**, **2m**, and **2j** were readily separated from catalytic Glu by ion exchange chromatography. Indeed, these analogues showed stronger interactions with basic Dowex-type resins presumably through hydrophobic interactions. The AAT-CSA regeneration system was preferred in the case of **2m** and **2p**. However, as **1j** is a substrate of AAT, the GluDH/NADH/FDH system and excess ammonium formate were used for the preparation of **2j**.

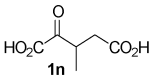
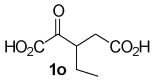
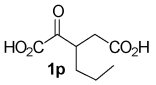
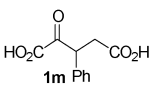
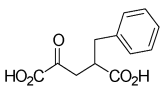
The results obtained for the different transamination assays are reported in Table 2.

In accordance with analytical results, mixtures of diastereomers were obtained in good yields from **1n**, **1o**, and **1j** substrates. In these three cases, a small scale transamination reaction was run and stopped near 45% conversion. As judged by NMR analysis, mixtures of diastereomers were obtained with de of <1%, 24%, and 31% from **1j**, **1n**, and **1o** respectively. These results confirmed the low enantiopreference of BCAT toward these substrates. On the contrary, 3-propyl and 3-phenyl Glu analogues **2p** and **2m** were both isolated as a single isomer. The (2*S*,3*R*) configuration was unambiguously attributed to the 3-phenyl analogue **2m** in comparison to the physicochemical properties and spectroscopic data previously described for this compound.[23,24] The new 3-propyl analogue **2p** possesses the L-2,3-*syn*-configuration. The (2*S*,3*R*)-configuration of **2p** was demonstrated by oxidative decarboxylation of the residual unreacted ketoacid **1p** after the BCAT catalyzed transamination. The known (*S*)-2-propylsuccinic acid **9** was identified,[25] thus showing that BCAT displays a high enantioselectivity toward (*R*)-**1p**.

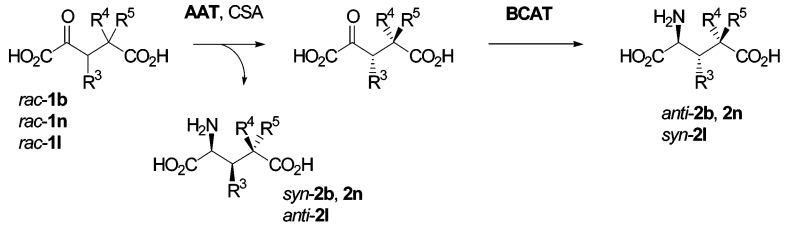
Finally, to exemplify the stereochemical complementarity of AAT and BCAT, we decided to develop a two-transamination-step strategy as described in Scheme 7 to prepare separately both stereomers of 3-methyl, 4-methyl, and 4-hydroxy-4-methyl-L-Glu **2n**, **2b**, and **2l**.

The racemic ketoacids **1b**, **1n**, and **1l** were first subjected to an AAT-catalyzed transamination reaction in the presence of CSA. The reaction was monitored by enzymatic titration of pyruvic acid formed from CSA using NADH and lactic dehydrogenase. The reaction was stopped near 50% conversion to perform the kinetic resolution of the racemic KG substrate. Indeed, as previously mentioned, AAT was shown to display a high enantiopreference for (*R*)-**1b** and for (*S*)-**1l.**[16,26] A high stereoselectivity was also observed in favor of (*R*)-**1n**. The amino acid product was selectively adsorbed on a short column of strongly acidic resin, and residual KG analogue was then submitted to a second transamination reaction catalyzed by

TABLE 2. Equilibrium-Shifted BCAT-Catalyzed Transamination of KG Analogues

Substrate	Amino donor	Regeneration system	Isolated yield (%)	Products stereochemistry	de (%)
 **1n**	Leu	LeuDH/NADH/FDH	73%	(2*S*,3*S*) and (2*S*,3*R*)	4
 **1o**	Leu	LeuDH/NADH/FDH	90%	(2*S*,3*S*) and (2*S*,3*R*)	4
 **1p**	Glu	AAT/CSA	25%	(2*S*,3*R*)	>95
 **1m** Ph	Glu	AAT/CSA	38%	(2*S*,3*R*)	>98
 **1j**	Glu	GluDH/NADH/FDH	59%	(2*S*,4*S*) and (2*S*,4*R*)	<1

SCHEME 7. Two-Step Transamination Process Using AAT and BCAT



Substrate	R^3	R^4	R^5	AAT transamination			BCAT transamination		
				Product	*Yield*	*de*	*Product*	*Yield*	*de*
rac-**1b**	H	Me	H	(2*S*,4*R*)-**2b**	47 %	88 %	(2*S*,4*S*)-**2b**	44 %	> 98 %
rac-**1n**	Me	H	H	(2*S*,3*R*)-**2n**	46 %	96%	(2*S*,3*S*)-**2n**	27 %	86%
rac-**1l**	H	Me	OH	(2*S*,4*S*)-**2l**	47 %	97%	(2*S*,4*R*)-**2l**	35 %	80%

BCAT. Leu and the LeuDH/NADH/FDH regeneration system were used in the case of **1n** as already described. Glu and AAT/CSA were used for **1l**; in that case, **2l** was easily separated from Glu on cationic Dowex 1 resin. Although the diastereomeric excess of (2*S*,4*R*)-**2l** isolated after the BCAT catalyzed reaction was only 80%, this chromatography allowed a partial separation of diastereoisomers of **2l** and an increase of the diastereomeric excess of (2*S*,4*R*)-**2l** up to 98%. Another strategy was tried with **1b**, by using 4-benzyl-Glu **2j** as the catalytic amino donor with the AAT/CSA regeneration system. Indeed, contrary to Glu, **2j** proved to be easily separated from 4-methyl Glu **2b** by anion exchange chromatography on Dowex 1 resin. Practically, catalytic amounts of 4-benzyl KG **1j**, BCAT, and AAT were added, after pH adjustment, to the crude mixture of CSA and residual (*S*)-**1b**, with neither compound being adsorbed on the strongly acidic resin. This procedure proved to be very efficient, and (2*S*,4*S*)-**2b** was thus isolated in good yield and with very good de.

Conclusion

We have developed a new method for the preparation of the keto acid substrates of aminotransferases. This method appeared efficient and general and should allow the synthesis of many new derivatives in the near future. We have shown that *E. coli* BCAT is a useful tool for the stereoselective preparation of glutamic acid analogues from the corresponding substituted ketoglutaric acids, especially 3-substituted derivatives that could not be obtained before with AAT. Furthermore, BCAT stereoselectivity is complementary to that of AAT and both enzymes offer access to both stereomers of a variety of L-glutamic acids substituted at position 4. These compounds were prepared with a high purity, suitable for biological evaluations.

Experimental Section

1-Methyl-5-ethyl 2-Methylidene-3-phenyl-glutarate 5. To a solution in anhydrous toluene (25 mL) of methyl 2-hydroxymethyl-3-phenylacrylate **4** (1.0 g, 5.2 mmol) prepared following a described procedure[20] were added triethyl orthoacetate (1.3 g, 7.8 mmol) and propionic acid (0.04 mL, 0.5 mmol). The mixture was heated under reflux for 1 h before methanol was slowly removed by azeotropic distillation. The solution was concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 9:1, v/v) afforded **5** (1.1 g, 80%), isolated as a colorless liquid: IR (neat film) 1728, 1709, 1633 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.32−7.20 (5H, m), 6.35 (1H, s), 5.69 (1H, s), 4.47 (1H, t, J = 8.0 Hz), 4.08 (2H, q, J = 7.0 Hz), 3.69 (3H, s), 2.93 (1H, dd, J = 8.0 and 15.5 Hz), 2.81 (1H, dd, J = 8.0 and 15.5 Hz), 1.18 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 166.7, 142.5, 141.2, 128.5, 127.9, 126.9, 124.5, 60.5, 52.1, 42.7, 39.6, 14.1. Anal. Calcd for $C_{15}H_{18}O_4$: C, 68.68, H, 6.92. Found: C, 68.52, H, 6.97.

1-Methyl-5-ethyl 3-Phenyl-2-oxoglutarate 6m. To a solution of **5** (1 g, 3.8 mmol) in a mixture of CCl_4 (10 mL) and CH_3CN (10 mL) were added a solution of $NaIO_4$ (3.24 g, 15.2 mmol) in water (15 mL) and RuO_2 (54 mg, 0.4 mmol). The mixture was stirred at room temperature for 24 h and then filtered on celite. The organic layer was isolated, and the aqueous phase was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 8:2, v/v) afforded **6m** (710 mg, 70%) isolated as a colorless liquid: IR (neat film) 1731 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 7.36−7.26 (5H, m), 4.98 (1H, dd, J = 5.0 and 10.5 Hz), 4.12 (2H, m), 3.79 (3H, s), 3.28 (1H, dd, J = 10.5 and 17.0 Hz), 2.72 (1H, dd, J = 5.0 and 17.0 Hz), 1.23 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6, 171.4, 160.7, 134.6, 129.2, 128.8, 128.2, 61.0, 53.0, 49.4, 37.1, 14.1. Anal. Calcd for $C_{14}H_{16}O_5$: C, 63.63, H, 6.10. Found: C, 63.49, H, 5.97.

Dilithium 3-Phenyl-2-oxoglutarate 1m. To a solution of **6m** (660 mg, 2.5 mmol) in MeOH (13 mL) was added dropwise a 0.4 M aqueous solution of LiOH (13 mL, 5.25 mmol). The mixture was stirred at room temperature for 3−4 h until complete hydrolysis. After evaporation of MeOH, the pH of the aqueous solution was adjusted to 7.6 by the addition of Dowex 50X8 resin (H^+ form). The resin was removed by filtration before evaporation of the water under reduced pressure. **1m** (575 mg, 98%) was isolated as a white solid: ^1H NMR (400 MHz, D_2O) δ 7.39−7.25 (5H, m), 4.66 (1H, t, J = 7.0 Hz), 2.89 (1H, dd, J = 7.0 and 15 Hz), 2.56 (1H, dd, J = 7.0 and 15 Hz); ^{13}C NMR (100 MHz, D_2O) δ 204.4, 180.0, 169.9, 136.5, 129.1, 128.7, 127.7, 51.8, 39.1; HRMS (TOF MS ES$^-$) m/z calcd for $C_{11}H_9O_5$ 221.0450, found 221.0472.

Dimethyl 3-Methyl-2-oxoglutarate 6n. A solution of **8n** (775 mg, 3 mmol) in CH_2Cl_2 (10 mL) was treated with a mixture of O_2 and O_3 bubbling at a rate of 10 L/h until saturation (blue coloration of the solution). After 30 min, the excess ozone was eliminated by oxygen bubbling. Dimethylsulfure (0.33 mL, 4.5 mmol) was added, and the reaction mixture was allowed to warm to room temperature. After dilution with CH_2Cl_2 (15 mL), the solution was washed with water (20 mL) and brine (20 mL), dried over $MgSO_4$, and

(23) Cai, C.; Soloshonok, V. A.; Hruby, V. J. *J. Org. Chem.* **2001**, *66*, 1339−1350.
(24) Herdeis, C.; Kelm, B. *Tetrahedron* **2003**, *59*, 217−229.
(25) Bettoni, G.; Cellucci, C.; Berardi, F. *J. Heterocycl. Chem.* **1980**, *17*, 603−605.
(26) Echalier, F.; Constant, O.; Bolte, J. *J. Org. Chem.* **1993**, *58*, 2747−2750.

concentrated under reduced pressure. Flash chromatography (eluent, CH_2Cl_2) afforded **6n** (0.33 g, 58%) isolated as a colorless liquid: IR (neat film) 1735 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.66 (1H, qdd, J = 5.0, 7.0 and 9.0 Hz),3.63 (3H, s), 2.81 (1H, dd, J = 9.0 and 17.0 Hz), 2.48 (1H, dd, J = 5.0 and 17.0 Hz), 1.18 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.8, 172.1, 161.2, 53.0, 51.9, 38.2, 36.8, 15.8. Anal. Calcd for $C_8H_{12}O_5$: C, 51.06, H, 6.43. Found: C, 51.34, H, 6.48. HRMS (TOF MS ES$^+$) *m*/*z* calcd for $C_8H_{12}O_5Na$ 211.0582, found 211.0594;

Dimethyl 2-Acetyl-3-methylglutarate 7n. To a solution of methyl crotonate (2.0 g, 20 mmol) and methyl acetoacetate (4.65 g, 40 mmol) in MeOH (40 mL) was added a 1 M solution of MeONa in MeOH (4 mL). The mixture was heated to reflux for 1 day before the addition of the MeONa solution was repeated (4 mL). After 1 more day of refluxing, methyl crotonate was added again (4.0 g, 40 mmol), and heating was continued for 1 day. After cooling, the solution was diluted with brine (50 mL) and extracted with EtOAc (4 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 7:3, v/v) afforded a 1:1 diastereomeric mixture of **7n** (5.7 g, 66%) isolated as a colorless liquid: IR (neat film) 1735 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.72 (3H, s), 3.71 (3H, s), 3.66 (2 × 3H, s), 3.56 (1H, d, J = 8.0 Hz), 3.48 (1H, d, J = 8.0 Hz), 2.74 (2 × 1H, m), 2.50−2.20 (2 × 2H, m), 2.23 (2 × 3H, s), 0.99 (2 × 3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5, 202.4, 172.4, 169.2, 63.69, 63.6, 52.30, 52.26, 51.5, 38.4, 38.2, 29.9, 29.7, 29.6, 17.7, 17.3; HRMS (TOF MS ES$^+$) *m*/*z* calcd for $C_{10}H_{16}O_5Na$ 239.0895, found 239.0897.

Dimethyl 2-(1-Acetoxyethylidene)-3-methylglutarate 8n. To a solution of **7n** (1.1 g, 5 mmol) in anhydrous pyridine (20 mL) was added acetyl chloride (1.1 mL, 15 mmol). The mixture was stirred at 45 °C for 3 days. Addition of acetyl chloride (0.55 mL, 7.5 mmol) was repeated at 24 and 48 h. After cooling, the solution was diluted with Et_2O (80 mL), washed with water (50 mL) and with an aqueous saturated solution of CuSO4 (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 8:2, v/v) afforded **8n** (0.83 g, 64%), isolated as a colorless liquid: IR (neat film) 1762, 1735, 1658 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.77 (3H, s), 3.65 (3H, s), 3.24 (1H, hex, J = 7.0 Hz), 2.54 (1H, dd, J = 6.0 and 15.0 Hz), 2.48 (1H, dd, J = 7.0 and 15.0 Hz), 2.20 (3H, s), 2.11 (3H, s), 1.09 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 168.2, 167.8, 153.1, 125.6, 51.5, 39.6, 29.1, 20.8, 19.1, 18.5; HRMS (TOF MS ES$^+$) *m*/*z* calcd for $C_{12}H_{18}O_6Na$ 281.1001, found 281.0997.

Dimethyl 3-Methyl-2-oxoglutarate 6n. A solution of **8n** (775 mg, 3 mmol) in CH_2Cl_2 (10 mL) was treated with a mixture of O_2 and O_3 bubbling at a rate of 10 L/h until saturation (blue coloration of the solution). After 30 min, the excess ozone was eliminated by oxygen bubbling. Dimethylsulfure (0.33 mL, 4.5 mmol) was added, and the reaction mixture was allowed to warm to room temperature. After dilution with CH_2Cl_2 (15 mL), the solution was washed with water (20 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (eluent, CH_2Cl_2) afforded **6n** (0.33 g, 58%) isolated as a colorless liquid: IR (neat film) 1735 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.66 (1H, qdd, J = 5.0, 7.0 and 9.0 Hz),3.63 (3H, s), 2.81 (1H, dd, J = 9.0 and 17.0 Hz), 2.48 (1H, dd, J = 5.0 and 17.0 Hz), 1.18 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.8, 172.1, 161.2, 53.0, 51.9, 38.2, 36.8, 15.8. Anal. Calcd for $C_8H_{12}O_5$: C, 51.06, H, 6.43. Found: C, 51.34, H, 6.48. HRMS (TOF MS ES$^+$) *m*/*z* calcd for $C_8H_{12}O_5Na$ 211.0582, found 211.0594;

(2*S*,3*R*)-3-Phenylglutamic Acid 2m. To a solution of **1m** (0.154 g, 0.66 mmol) in water (45 mL) were added cysteine sulfinic acid (CSA) (100 mg, 0.66 mmol), L-glutamic acid (19 mg, 0.13 mmol), and a 10 mM solution of pyridoxal phosphate (0.5 mL, 0.5 μmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH, and the volume was adjusted to 50 mL before *E. coli* BCAT (2 IU) and *E. coli* AAT (10 IU) were added. The reaction was stirred slowly at room temperature and monitored by titration of pyruvate: 5 μL aliquots of the reaction mixture were added to 995 μL of 0.1 M potassium phosphate buffer, pH 7.6 containing NADH (0.2 mM), and rabbit muscle lactate dehydrogenase (1 IU). Pyruvate concentration was calculated from the ΔDO measured at 340 nm using ϵ_{NADH} = 6220 M^{-1} cm^{-1}. When a conversion rate of 40% was reached, the reaction mixture was rapidly passed through a column of Dowex 50X8 resin (H^+ form, 25 mL). The column was then washed with water (100 mL) until complete elution of CSA and then eluted with 1 M NH_4OH. The ninhydrin positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted in water (5 mL), and the pH was adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of Dowex 2X8 resin (200−400 Mesh, AcO^- form, 1.5 cm × 12 cm). The column was washed with water (50 mL) and then eluted with an AcOH gradient (0.1−0.5M). L-Glutamic acid was eluted first and completely separated from (2*S*,3*R*)-**2m** isolated as a white solid (56 mg, 38% from *rac*-**1m**) and with a diastereomeric excess over 98% as judged by NMR: mp 160 °C (lit. mp 158.0−158.5 °C[23], 197 °C[24]); $[\alpha]^{25}_D$ = +16.9° (*c* 0.5, 6 N HCl) (lit. $[\alpha]^{25}_D$ = +16.7° (*c* 1.36, 6 N HCl)[23]; +16.7° (*c* 0.6, 6 N HCl)[24]); ^1H NMR (400 MHz, D_2O, LiOH) δ 7.32−7.20 (5H, m), 3.67 (1H, d, J = 6.5 Hz), 3.46 (1H, m), 2.62 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 179.6, 174.6, 138.0, 128.9, 128.3, 127.8, 59.9, 44.1, 40.3. Anal. Calcd for $C_{11}H_{13}NO_4$, 1.2 H_2O: C, 53.96, H, 6.34, N, 5.72. Found: C, 54.14, H, 6.28, N, 5.66.

(2*S*,3*R*)- and (2*S*,3*S*)-3-Methylglutamic Acid 2n. Method A: Using *E. coli* BCAT. To a mixture of **1n** (50 mg, 0.29 mmol), L-leucine (4 mg, 0.03 mmol), NADH (2.0 mg, 0.003 mmol), and ammonium formate (36 mg, 0.58 mmol) in H_2O (10 mL) at pH 7.6 were added a 10 mM solution of pyridoxal phosphate (0.1 mL, 0.1 μmol), BCAT (1 IU), leucine dehydrogenase (2 IU), and formate dehydrogenase (3 IU). The solution was stirred at room temperature and monitored by titration of **1n**: 5 μL aliquots of the reaction mixture were added to 995 μL of 0.1 M potassium phosphate buffer, pH 7.6 containing L-glutamic acid (40 mM), $(NH_4)_2SO_4$ (50 mM), NADH (0.2 mM), BCAT (0.04 IU), and glutamic dehydrogenase (2 IU). The concentration of **1n** was calculated from the ΔDO measured at 340 nm using ϵ_{NADH} = 6220 M^{-1} cm^{-1}. After 24 h the reaction mixture was passed through a column of Dowex 50X8 resin (H^+ form, 10 mL). The column was washed with water (30 mL) and then eluted with 1 M NH_4OH. The ninhydrin positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted with water (5 mL), and the pH was adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of Dowex 2X8 resin (200−400 mesh, AcO^- form, 1.5 cm × 12 cm). The column was washed with water (50 mL) and then eluted with 0.2 M AcOH. **2n** was isolated as a white solid (34 mg, 73%) as a 48:52 mixture of (2*S*,3*R*) and (2*S*,3*S*) diastereomers as determined by ^1H NMR.

Method B: Using AAT and BCAT. To a mixture of **1n** (100 mg, 0.58 mmol) and CSA (90 mg, 0.58 mmol) in water (20 mL) at pH 7.6 was added AAT (50 IU). The reaction mixture was stirred at room temperature and monitored by titration of **1n** as previously described in method A. When a conversion rate near 50% was reached in 32 h, the reaction mixture was passed through a column of Dowex 50X8 resin (H^+ form, 10 mL). The column was washed with water (30 mL), and the fractions containing unreacted (*S*)-**1n** were pooled and concentrated under reduced pressure after adjusting the pH to 7.6 with 1 M NaOH. The column was then eluted with 1 M NH_4OH, and (2*S*,3*R*)-**2n** was further purified and isolated as previously described in method A (43 mg, 46%) with a diastereomeric excess of 96% as judged by ^1H NMR. Crude residual (*S*)-**1n** was used in a BCAT-catalyzed transamination reaction as described previously in method A. (2*S*,3*S*)-**2n** was isolated as a white solid (25 mg, 27%) with a diastereomeric excess of 86%.

Data for (2*S*,3*R*)-2n: mp 161−163 °C (lit. mp 166.0−168 °C[27], 170−173 °C[24]); $[\alpha]^{25}_D = +18.2$ (*c* 0.9, 6 N HCl) (lit. $[\alpha]^{25}_D = +22.6$ (*c* 1.03, 6 N HCl)[27]); ^{1}H NMR (400 MHz, D_2O, 1 M NaOH) δ 3.14 (1H, d, $J = 4.0$ Hz), 2.22 (1H, dd, $J = 5.0$ and 13.0 Hz), 2.18 (1H, m), 1.98 (1H, dd, $J = 9.5$ and 13.0 Hz), 0.79 (3H, d, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, D_2O) δ 182.4, 182.3, 60.1, 42.5, 34.9, 13.7. Anal. Calcd for $C_6H_{11}NO_4$, 0.33 H_2O: C, 43.13, H, 7.03, N, 8.38. Found: C, 43.01, H, 6.99, N, 8.25.

Data for (2*S*,3*S*)-2n: mp 171−173 °C (lit. mp 169.5.0−170 °C[28], 169−171 °C[27], 182−184 °C[24]); $[\alpha]^{25}_D = +42.0$ (*c* 0.9, 6 N HCl) (lit. $[\alpha]^{25}_D = +41.9$ (*c* 1.03, 6 N HCl),[24] +42.8 (*c* 1.0, 6 N HCl),[28] +36.8 (*c* 1.0, 6 N HCl)[27]); ^{1}H NMR (400 MHz, D_2O, 1 M NaOH) δ 2.96 (1H, d, $J = 6.0$ Hz), 2.26 (1H, dd, $J = 4.0$ and 13.5 Hz), 1.96 (1H, m), 1.79 (1H, dd, $J = 11.0$ and 13.0 Hz), 0.81 (3H, d, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, D_2O) δ 182.6, 182.3, 61.3, 41.0, 35.5, 16.0. Anal. Calcd for $C_6H_{11}NO_4$, 0.66 H_2O: C, 41.64, H, 7.18,N, 8.09. Found: C, 41.42, H, 6.72,N, 7.86.

(2*S*,4*R*)- and (2*S*,4*S*)-4-Methylglutamic Acid 2b. To a mixture of **1b** (300 mg, 1.74 mmol) and CSA (266 mg, 1.74 mmol) in water (90 mL) at pH 7.6 was added AAT (40 IU). The reaction mixture was stirred at room temperature and monitored by titration of pyruvate as previously described for the synthesis of **1m**. When a conversion rate near 50% was reached in 2 h, the reaction mixture was passed through a column of Dowex 50X8 resin (H^+ form, 10 mL). The column was washed with water until complete elution of excess CSA. The fractions containing unreacted (*S*)-**1b** and CSA were pooled and concentrated under reduced pressure after adjusting the pH to 7.6 with 1 M NaOH. The column was then eluted with 1 M NH_4OH, and (2*S*,4*R*)-**2b** was isolated and further purified on Dowex 2 resin as previously described for **2n**. (2*S*,4*R*)-**2b** was isolated as a white solid (132 mg, 47%) with a diastereomeric excess of 88% as judged by ^{1}H NMR. Physical and spectroscopic properties were consistent with the previously reported data.[15] Anal. Calcd for $C_6H_{11}NO_4$, 0.33 H_2O: C, 43.13, H, 7.03, N, 8.38. Found: C, 43.24, H, 6.89, N, 8.75.

To the solution of residual (*S*)-**1b** and CSA in water (30 mL) were added cysteine sulfinic acid (CSA) (75 mg, 0.5 mmol), dilithium 4-benzyl-2-oxoglutarate **1j** (25 mg, 0.1 mmol), and a 10 mM solution of pyridoxal phosphate (0.45 mL, 4.5 μmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH, and the volume was adjusted to 45 mL before BCAT (5 IU) and AAT (10 IU) were added. The reaction was stirred slowly at room temperature for 15 h. The reaction mixture was passed through a column of Dowex 50X8 resin (H^+ form, 25 mL). The column was then washed with water (100 mL) until complete elution of excess CSA and then eluted with 1 M NH_4OH. The ninhydrin positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted in water (5 mL), and the pH was adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of Dowex 2X8 resin (200−400 mesh, AcO^- form, 1.5 cm × 12 cm). The column was washed with water (50 mL) and then eluted with 0.2 M AcOH. The ninhydrin positive fractions were concentrated under reduced pressure to give (2*S*,4*S*)-**2b** (123 mg, 44%) isolated as a white solid and with a diastereomeric excess over 98%. Physical and spectroscopic properties of (2*S*,4*S*)-**2b** were consistent with the previously reported data.[26] Anal. Calcd for $C_6H_{11}NO_4$, 0.33 H_2O: C, 43.13, H, 7.03, N, 8.38. Found: C, 42.77, H, 6.91,N, 8.83. Consecutive elution of the column with 2 M AcOH afforded an equimolar diastereomeric mixture of **2j** (20 mg).

(2*S*,4*R*)- and (2*S*,4*S*)-4-Benzylglutamic Acid 2j. To a mixture of **1j** (50 mg, 0.2 mmol), l-glutamic acid (6 mg, 40 μmol), NADH (1.5 mg, 2 μmol), and ammonium formate (32 mg, 0.4 mmol) in H_2O (10 mL) at pH 7.6 were added a 10 mM solution of pyridoxal phosphate (0.1 mL, 0.1 μmol), BCAT (2 IU), glutamic dehydrogenase (3 IU), and formate dehydrogenase (4 IU). The solution was stirred at room temperature and monitored by titration of **1j** as previously described for the synthesis of **2n**, method A. After 3 h, the reaction was stopped ,and **2j** was purified as described for **2m**; **2j** was isolated as a white solid (28 mg, 59%) and as a 1:1 mixture of (2*S*,4*R*) and (2*S*,4*S*) stereomers: ^{1}H NMR (400 MHz, D_2O) δ 7.40−7.20 (2 × 5H, m), 3.72 (1H, dd, $J = 5.5$ and 8.5 Hz), 3.68 (1H, dd, $J = 6.0$ and 8.5 Hz), 3.00−2.80 (2 × 3H, m), 2.22 (1H, ddd, $J = 5.5$, 9.5 and 15.0 Hz), 2.08 (1H, ddd, $J = 6.5$, 10.0 and 15.0 Hz), 1.96 (1H, ddd, $J = 4.5$, 8.0 and 14.5 Hz), 1.92 (1H, ddd, $J = 4.5$, 9.0 and 15.0 Hz); ^{13}C NMR (100 MHz, D_2O , LiOH) δ 183.6, 183.3, 140.3, 140.2, 128.9, 128.4, 126.2, 48.2, 48.0, 38.8, 38.3, 37.4, 37.0. Anal. Calcd for $C_{12}H_{15}NO_4$, 0.33 H_2O: C, 59.25, H, 6.49, N, 5.76. Found: C, 59.20, H, 6.46, N, 5.60.

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Supporting Information Available: General experimental information, enzymatic assays procedures, characterization of compounds **1n−p**, **2o,p**, **6o,p**, **7o,p**, **8o,p**, and **9**, copies of ^{1}H and ^{13}C NMR spectra for compounds **1m−p**, **2b−p**, **5**, **6m−p**, **7n−p**, **8n−p**, and **9**. This material is free of charge via the Internet at http://pubs.acs.org.

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